THE SELECTIVE INHIBITORY ACTION OF METHYLENE BLUE AND CERTAIN OTHER COMMON DYES ON THE GROWTH OF MENINGOCOCCI

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In an effort to demonstrate the presence of capsules in certain strains of meningococci it was observed that safranin added to suspensions of the bacteria caused agglutination and subsequent settling of the suspended organism. Further investigation of this phenomenon showed that agglutination and complete precipitation occurred in dilutions of 1:2,000 and partial agglutination in dilutions of 1:10,000. This was first suspected of being a specific phenomenon, but the action of safranin on suspensions of B. typhosus and Streptococcus viridans revealed a similar agglutination. Subsequently it was found that the dye added in like dilutions to infusion broth caused a flocculent coagulation of protein closely resembling what had been regarded as agglutination. It may be assumed, perhaps, that the agglutination of suspended organisms caused by sanfranin is really due to protein coagulation. A further study of the action of safranin or suspensions of meningococci showed inhibition of their growth at dilutions between 1:1,000 and 1:10,000. This led to an investigation of the behavior of certain other common dyes both on meningococci and other pathogenic organisms, and a comparison of their inhibitory action with that of a few of the common antiseptics.

All meningococci used were obtained from the spinal fluids of cases of epidemic cerebrospinal meningitis among troops in France. Where the type of meningococcus is given it was established on the basis of the French classification, using immune type serum obtained from the Pasteur Institute. Meningococci were cultivated on Kinnicutt's coagulated sheep blood medium — defibrinated sheep blood, 2 or 3 parts; dextrose infusion broth (0.2 acid to phenolphthalein), 1 part. This is an admirable medium for meningococci. Growth usually takes place in 24 hours, the organisms forming circular, raised, shiny, grayish colonies of characteristic appearance. Meningococci cultivated on this medium retain their viability at incubator temperature for a month or more, when rubber stoppers are used to prevent evaporation and drying.

One per cent. solutions of the dyes were made in sterile distilled water using sterile glassware. The solutions were then arnoldized. From 1% solutions dilutions were made 10 times stronger than the ultimate dilutions desired. The ultimate dilutions were obtained by adding 9 parts of bacterial suspensions to 1 part of diluted dye. In the majority of experiments the series of dilutions were set up in small test tubes measuring approximately 6 by 1.2 cm. In these tubes 0.1 c c diluted dye and 0.9 c c suspensions were mixed. In some experiments larger volumes were employed, namely, 1 c c dye and 9 c c suspension.

Suspensions of approximately the same density were made in sterile normal salt solution from organisms grown on solid mediums.

The mixtures of dye and bacterial suspension were allowed to remain at incubator temperature for one hour. They were then planted by pouring the entire contents of the tubes into culture tubes of slanted solid medium, from which the water of condensation had been poured off. By tilting the culture tubes the dye suspension mixtures were flowed over the surface of the medium three times. They were then incubated in the upright

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position without permitting dye suspension mixtures to come in contact with slanted surface again. Cultures were examined for growth after 24-48 hours.

A more desirable technic would have been to cultivate the organisms directly in fluid medium containing dyes at various dilutions, were it not for the aerobic habits of the meningococcus; growth in fluid medium being scant and capricious.

In each experiment the use of a control tube containing 1 part of distilled water (in place of dye) and 9 parts of bacterial suspension demonstrated that the organisms retained their viability under the conditions of the experiment.

Exper. 1.—Comparative inhibitory action of gentian violet, safranin, methylene blue, fuchsin, eosin and phenol on meningococcus, strain 4S, type "B." Suspensions made with 60 c c normal saline solution from three 42-hour cultures in Pasteur tubes. Dye suspension mixtures in incubator 1 hour and twenty minutes before planting.

Dilutions	Gentian Violet	Safranin	Methylene (Leitz) Blue	Fuchsin (Basic)	Eosin	Phenol
1: 1,000.....	—	—	--	±	+	+
1: 2,000.....	—	—	±	+	+	+
1: 4,000.....	—	+	±	+	+	+
1: 8,000.....	—	+	±	+	+	+
1: 10,000.....	—	+	+	+	+	+
1: 20,000.....	—	+	+	+	+	+
1: 40,000.....	—	+	+	+	+	+
1: 80,000.....	+	+	+	+	+	+
Control.....	+	+	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.
± indicates a very slight growth after 48 hours' incubation, recognizable only by aid of a hand lens.
— indicates no macroscopic growth after 48 hours' incubation.

Exper. 2.—Comparative inhibitory action of crystal violet, brilliant green, bismarck brown, methylene blue (American and French preparations), vital red and fluorescein on meningococcus strain 4S, type "B." Suspension made with about 60 c c normal saline from one 48-hour culture in Pasteur tube. Dye suspension mixture in incubator 1 hour and 15 minutes before planting.

Dilutions	Crystal Violet	Brilliant Green	Bismarck. Brown	Methylene Blue (American)	Methylene Blue (French)	Vital Red	Fluorescein
1: 1,000.....	..	..	..	—	—	+	+
1: 2,000.....	—	—	—	—	—	+	+
1: 4,000.....	—	—	—	—	—	+	+
1: 8,000.....	—	—	—	—	+	+	+
1: 10,000.....	—	—	—	—	+	+	+
Control.....	+						

Examination of these results shows that at the dilutions employed several of the dyes had a marked inhibitory action on the growth of this strain of meningococcus, whereas others had little or none. Because of its relative lack of toxicity it was determined to investigate further the action of methylene blue.

Exper. 3.—Comparative inhibitory action of methylene blue on meningococci, types "A," "B" and "C," and various other pathogenic organisms. Suspensions

made in sterile saline and diluted to approximately the same density. All cultures except B. typhosus 30 hours old. B. typhosus culture 10 hours. Dye suspension mixtures in incubator 1 hour before planting.

Dilutions of Methylene Blue (Leitz)	Meningococcus Type "A"	Meningococcus Type "B"	Meningococcus Type "C"	Bacillus Typhosus	Bacillus Coli	Bacillus Dysenteriae	Bacillus Pyocyaneus	Staphylococcus Albus	Streptococcus Viridans	Streptococcus Hemolyticus	Bacillus Diphtheriae	Pneumococcus Group III
1: 1,000	—	—	—	+	+	+	+	+	+	+	+	+
1: 2,000	—	—	—	+	+	+	+	+	+	+	+	+
1: 4,000	—	±	—	+	+	+	+	+	+	+	+	+
1: 8,000	—	+	—	+	+	+	+	+	+	+	+	+
1: 10,000	±	+	—	+	+	+	+	+	+	+	+	+
1: 20,000	+	+	—	+	+	+	+	+	+	+	+	+
1: 30,000	+	+	—	+	+	+	+	+	+	+	+	+
1: 40,000	+	+	—	+	+	+	+	+	+	+	+	+
1: 50,000	+	+	—	+	+	+	+	+	+	+	+	+
Control	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.
± indicates a very slight growth after 48 hours' incubation, recognizable only by aid of a hand lens.
— indicates no macroscopic growth after 48 hours' incubation.

Under the conditions of this experiment inhibition of growth occurred in cultures of the three strains of meningococci alone and in none of the other pathogenic organisms.

To determine the behavior of methylene blue on an organism biologically related to meningococcus a strain of gonococcus was obtained in pure culture and comparative titrations made with it and two strains of meningococci.

Exper. 4.—Comparative inhibitory action on gonococcus and on meningococci strain 4S, type "A," and strain 31S, type undetermined. Suspensions of gonococcus made with 10 c c sterile saline from 3 Kinnicutt's coagulated sheep blood cultures, respectively, 48 hours, 4 and 5 days old. Suspensions of meningococci for each strain made with 60 c c sterile saline from 48-hour Pasteur tube culture. Dye suspension mixtures in incubator 1 hour and 15 minutes before planting.

Dilutions of Methylene Blue (French)	Gonococcus	Meningococcus 4S	Meningococcus 31S
1: 1,000	—	—	—
1: 2,000	—	—	—
1: 4,000	—	—	—
1: 8,000	—	—	+
1: 10,000	—	—	+
Control	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.
— indicates no macroscopic growth after 48 hours' incubation.

In these experiments the action of methylene blue on the gonococcus and meningococcus showed a correspondence which was at variance with its action on the other pathogenic organisms tried.

From the tables it will be observed that the dilution of methylene blue at which growth capacity of the meningococcus is lost is variable. Even with constant conditions of medium, age of culture and time of incubation of dye suspension mixtures, there are a number of variables which no doubt contribute to establish this point. An important and obvious one is the number of organisms present in a suspension. To test this the action of methylene blue on a suspension was compared with its action on one made by diluting the first suspension 10 times.

Exper. 5.—Comparative inhibitory action of methylene blue on meningococcus strain 1S, type "A," and on B. typhosus suspensions of varying density. Original suspensions made with 20 c c sterile saline from 79-hour culture of meningococcus and 20-hour B. typhosus. Original suspension diluted by 10. Dye suspension mixtures in incubator 1 hour before planting.

Dilutions of Methylene Blue (Leitz)	Meningococcus Suspension	Meningococcus Suspension Diluted x10	Bacillus Typhosus Suspension	Bacillus Typhosus Suspension Diluted x10
1: 10,000	—	—	+	+
1: 20,000	+	—	+	+
1: 30,000	+	—	+	+
1: 40,000	+	—	+	+
1: 50,000	+	—	+	+
1: 60,000	+	—	+	+
1: 70,000	+	+	+	+
1: 80,000	+	+	+	+
1: 90,000	+	+	+	+
1: 100,000	+	+	+	+
1: 150,000	+	+	+	+
1: 200,000	+	+	+	+
1: 300,000	+	+	+	+
Control	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.
— indicates no macroscopic growth after 48 hours' incubation.

A variation between 1: 10,000 and 1: 60,000 is seen here in the inhibitory point of methylene blue on the growth of this strain of meningococcus, depending on the number of organisms present in the suspension. With B. typhosus no inhibition occurred even in the dilute suspension.

To determine whether for a given suspension the inhibitory point of methylene blue is constant, an experiment was made using three strains of meningococci. The suspensions made from each were titrated in triplicate. An inhibitory point was found different for the three strains, but constant for each set of triplicates.

Exper. 6.—Comparative inhibitory action of methylene blue on strains of meningococci 4S, 30S and 31S, each one titrated in triplicate. Suspensions made with 60 c c sterile saline from one 48-hour Pasteur tube culture each of 4S and 31S and with 30 c c sterile saline from a similar culture of 30S. Dye suspension mixtures in incubator for 1 hour and 45 minutes.

Dilutions of Methylene Blue (French)	4S			30S			31S		
	1	2	3	1	2	3	1	2	3
1: 1.000	—	—	—	—	—	—	—	—	—
1: 2,000	—	—	—	±	±	±	—	—	—
1: 4,000	—	—	—	+	+	+	—	—	—
1: 8 000	+	+	+	+	+	+	—	—	±*
1: 10,000	+	+	+	+	+	+	—	—	—
Control	+	+	+	+	+	+	+	+	+

+ indicates a macroscopic growth after 48 hours' incubation.
± indicates a very slight growth after 48 hours' incubation, recognizable only by aid of a hand lens.
— indicates no macroscopic growth after 48 hours' incubation.
* In this tube after 48 hours' incubation 3 colonies appeared on the slanted surface.

Because of the difficulty in obtaining suspensions of different strains of meningococci with equal numbers of organisms present, it seemed impracticable to attempt to express the inhibitory action of methylene blue on the meningococcus in any terms equivalent to a phenol coefficient. For one particular strain, however, a comparative study of the action of methylene blue and certain common disinfectants seemed of interest.

Exper. 7.—Comparative inhibitory action of formaldehyd, mercuric chlorid, phenol and methylene blue on meningococcus strain 4S, type "B." Suspensions made from four 24-hour cultures in Pasteur tubes.

Dilutions	Methylene Blue (French)	Formalin	Mercuric Chlorid	Phenol
1 : 1.000	—	—	—	+
1 : 2,000	—	—	—	+
1 : 4,000	+	+	—	+
1 : 8 000	+	+	—	+
1 : 10,000	+	+	—	+
Control	+			

+ indicates a macroscopic growth after 48 hours' incubation.
— indicates no macroscopic growth after 48 hours incubation (with phenol, inhibition occurred in a dilution of 1:100).

With this particular suspension the inhibitory point of methylene blue corresponded to that of a solution of formalin.

To investigate what influence the presence of protein had on the inhibitory action of methylene blue the following experiment was done.

Exper. 8.—Action of methylene blue in various dilutions on meningococcus in presence of leukocytes and native protein of spinal fluid.

About 60 c c of turbid spinal fluid from a fresh case of cerebrospinal meningitis was collected in a large test tube. Approximately ⅓ of this was planted by pouring into a flat-sided Pasteur tube containing the coagulated sheep blood medium. This and the remainder of the spinal fluid were incubated. After 48 hours a heavy growth appeared on the coagulated sheep blood. A suspension was made from this using the rest of the incubated spinal fluid to suspend the growth, in place of normal saline. The whole was thoroughly agitated to insure an equal distribution of organisms and sedimented pus cells. It was then added to varying dilutions of methylene blue in the proportion of 9 c c suspension to 1 c c of dye. The dye suspension mixtures were incubated for 1 hour and then poured into the Pasteur tubes containing the medium.

The accompanying table shows the result of this experiment. The presence of the native protein of inflammatory spinal fluid apparently exerted no influence on the inhibitory action of methylene blue.

Dilution of Methylene Blue (Leitz) in Spinal Fluid	Meningococcus Strain 308. Type Not Determined
1 : 2,000	—
1 : 4,000	—
1 : 8,000	—
1 : 10,000	—
Control	+

+ indicates a macroscopic growth after 48 hours' incubation.
— indicates no macroscopic growth after 48 hours incubation.

SUMMARY AND DISCUSSION

Of dyes studied, the following were found to inhibit the growth of meningococci: gentian violet, crystal violet, brilliant green, bismarck brown, safranin, methylene blue. In contrast to the above, basic fuchsin, vital red, fluorescein and eosin had no inhibitory action.

The study of the comparative action of methylene blue on various types of meningococci and other pathogenic organisms showed that the growth of meningococci was inhibited at dilutions which failed to inhibit the growth of the other organisms with one exception.

The inhibitory action of methylene blue on the meningococcus and the gonococcus, biologically related organisms, was the same.

No fixed point of dilution has been established at which methylene blue inhibits the growth of different suspensions of meningococci. This varies with the number of viable organisms present in the suspensions.

For a given suspension, however, the inhibitory point is constant.

A study of the comparative action of methylene blue, formaldehyd, mercuric chlorid and phenol showed mercuric chlorid to exert the